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"The gene cluster involved in aclacinomysin biosynthesis, and its use for genetic engineering"
(Aklasinomysiinin biosynteesiin liittyvä geeniryhmittymä ja sen käyttö-geenitekniikassa)

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The gene cluster involved in aclacinomycin biosynthesis, and its use for genetic engineering

Field of the invention

This invention relates to the gene cluster for aclacinomycin biosynthesis derived from *Streptomyces galilaeus*, and the use of the genes included therein to obtain hybrid antibiotics, or to increase yields of aclacinomycins or related antibiotics.

Background of the invention

Anthracyclines are widely used anticancer agents. Seven different anthracyclines are in worldwide clinical use: daunorubicin, doxorubicin, idarubicin, epirubicin, pirarubicin, zorubicin and aclarubicin. A representative compound is doxorubicin, being the most efficient and acting on a wide array of malignancies. A variety of toxic effects, like cumulative cardiotoxicity found with doxorubicin has sometimes led to discontinuation of the treatment. Furthermore, there are some type of malignancies which do not respond to available anthracyclines. The mechanism of action of anthracyclines, reflecting to their clinical efficiencies, is not clear, although most researchers consider inhibition of topoisomerase II as a desired effect. Generation of free radicals derived from quinonic structures is suggested to be related to side effects such as cardiotoxicity. Anthracyclines have recently been reviewed by Professor Strohl and his group (1997).

Aclacinomycin A (aclarubicin) first described by Oki *et al.* (1975) is an anthracycline antibiotic produced by *Streptomyces galilaeus* ATCC 31133 and *S. galilaeus* ATCC 31615. It is active against tumor cells and exhibits alleviated toxic properties as compared with doxorubicin. However, its activity does not reach solid tumors, limiting its use in leukemia treatment. Aclarubicin differs from the other counterparts in its structure. A trisaccharide moiety, rhodosamine–2–deoxyfucose–cinerulose A is attached at C–7 by a glycosidic bond, whereas at the corresponding position of daunomycins only one sugar residue, daunosamine, is attached.

Despite the long history of anthracyclines, three decades or so, the studies on their biosynthesis are still going on, and there is further interest to obtain novel molecules for

the development of cancer chemotherapeutics. A method currently used for finding novel molecules for drug screening is genetic engineering. Cloning the genes for anthracycline biosynthesis facilitates the production of hybrid anthracyclines, as well as their use in combinatorial biosynthesis to generate novel molecules. As regards the chemical nature of anthracyclines currently in clinical use, aclarubicin has unique features which make its biosynthetic genes interesting in creating novel products.

Regarding the genes for deoxyhexose pathway, Madduri *et al.* (1998) have reported that a gene derived from avermectin biosynthesis cluster caused the production of hybrid anthracyclines altering a sugar moiety when transferred into a *S. peucetius* strain. The product obtained was epirubicin, a commercially important anthracycline. In this case a hydroxy group in the daunosamine moiety was in the opposite stereochemistry due to the action of an avermectin biosynthesis gene.

S. galilaeus has been used as the host to prepare hybrid anthracyclines using the genes derived from rhodomycin pathway from *S. purpurascens* (Niemi *et al.,* 1994) and from nogalamycin biosynthesis cluster from *S. nogalater* (Ylihonko *et al.,* 1996a). The genes for nogalamycin pathway were used to generate the hybrid anthracycline production in *S. steffisburgensis* producing typically steffimycin (Kunnari *et al.,* 1997). Previously, biosynthesis genes for actinorhodin have been expressed in *S. galilaeus*, resulting in the formation of aloesaponarin (Strohl *et al.,* 1991). These hybrid compounds were modified in the aglycone moiety. Recently, the biosynthesis genes involved in deoxyhexose pathway of nogalamycin were used to generate hybrid compounds using the *S. galilaeus* mutants as hosts (FI pat. appln No. 982295).

As shown above, *S. galilaeus* has been used as a cloning host to generate novel molecules, whereas its use to donate the genes has not been described. The identified genes involved in aclacinomycin biosynthesis include polyketide reductase gene (Tsukamoto *et al.,* 1994), aklanonic acid methyl ester cyclase (GeneBank, ACCESSION AF043550) and genes for polyketide synthase (Hutchinson and Fujii, 1995; the sequence not available).

Summary of the invention

The present invention concerns a gene cluster, most of the genes of which are derived from deoxyhexose pathway for rhodosamine, 2–deoxyfucose and/or rhodinose. The gene cluster was cloned from *S. galilaeus* ATCC 31615 and it is involved in biosynthesis of aclacinomycins.

Detailed description of the invention

The experimental procedures of the present invention include biochemical and chemical methods conventional in the art. Detailed description of the techniques not explained here are given in the manuals by Hopwood *et al.* 'Genetic manipulation of Streptomyces: a laboratory manual'. The John Innes Foundation, Norwich (1985) and by Sambrook *et al.* (1989) 'Molecular cloning: a laboratory manual'.

The publications, patents and patent applications cited herein are given in the reference list in their entirety.

The present invention concerns particularly the discovery of the gene cluster for aclacinomycin biosynthesis. The cluster, when introduced into *S. peucetius* strains caused the production of hybrid antibiotics modified in their sugar moiety.

Several strategies may be adopted to clone genes for an antibiotic. Using *E. coli* as a host for a gene library, hybridization is the most advantageous screening strategy. The probe for hybridization may be any known fragment that shows sufficient homology to the biosynthetic cluster for aclarubicin sugars, to be able to hybridize with said cluster. A DNA fragment which is identical to the desired region is preferred. Such a fragment, called Sg–dht, was obtained by PCR amplification of *S. galilaeus* chromosomal DNA, using degenerated oligonucleotides annealing to the conserved region of 4,6–dehydratase gene. 4,6–dehydratase is the first enzyme participating to a reaction series that converts a glucose molecule bound to a nucleotide into 6–deoxy sugars generally found in antibiotics. Using this probe it was possible to clone the cluster of deoxyhexose pathway

from a restricted gene library. To simplify the cloning strategy the library was prepared in a pUC-based plasmid (e.g. pBluescript or pWHM1109) replicating in *E. coli*.

The strategy to clone the genes involved in aclacinomycin biosynthesis according to the invention was in brief: Total DNA was isolated from *S. galilaeus* (ATCC 31615) and digested with several restriction enzymes that yield fragments of 10 kb in average. Restriction fragments were analyzed by Southern hybridization using a homologous DNA fragment, Sg-dht, as a probe. *Bgl*II gave a hybridized fragment of 8.5 kb, and a double digestion with *Xho*I and *Not*I gave a hybridized fragment of 7 kb. DNA digestion using (i) *Bgl*II and (ii) *Xho*I-*Not*I was carried out and the fragments were ligated to the *E. coli-Streptomyces* shuttle vector, pWHM1109, digested with *Bam*HI and to the pBluescript digested with *Xho*I-*Not*I, respectively. The ligation mixtures were introduced into *E. coli* XL1BlueMRF' that exhibits alleviated restriction-modification systems. Colonies were plated on the agar plates in the dilution to give 200 to 600 cfu (colony forming units) per plate. Well grown colonies were transferred in nylon membranes for hybridization, which was carried out using the Sg-dht probe. Six out of the 786 *Bgl*II-digested clones gave hybridization signal and 7 out of 1523 of those clones carrying *Xho*I-*Not*I fragments. Hybridization and washes were carried out in the stringent conditions of 65°C in a low salt concentration. Several techniques for the labeling of the probe and for hybridization are possible, but the procedure according to Boehringer Mannheim's "The DIG System User's Guide for Filter Hybridization" is preferred. The colonies giving hybridization signals were cultivated for plasmid isolation. The plasmids were analyzed by Southern hybridization to confirm the reliability of the colony hybridization. Plasmids containing the desired DNA fragments (Sg4 and Sg5) were designated as pSgc4 (*Bgl*II-fragment) and pSgc5 (*Xho*I-*Not*I fragment)(see Fig. 2).

The fragments, Sg4 and Sg5, were subcloned for sequencing in *E. coli* vectors pUC19 and pBluescript. In total 30 subclones were used to obtain the nucleotide sequence of Sg4 and Sg5. The sequenced cluster revealed thirteen genes involved in biosynthesis of aclacinomycins. Comparison with the sequences found in the sequence library suggested the functions as *sga*2 for an activator, *sga*3 for a dehydratase, *sga*4 for oxidoreductase, *sga*5 for dTDP-glucose 4,6-dehydratase, *sga*6 for glycosyl transferase (GTF), *sga*7 for

a putative isomerase, *sga*8 for aklaviketone reductase, *sga*9 for a putative polyketide assembler, *sga*10 for a putative cyclase, *sga*11 for aminomethylase, *sga*12 for glucose–1–phosphate thymidylyl transferase, *sga*13 for aminotransferase. The function of *sga*1 is not suggested based on similarity searches. Based on the deduced functions, nine genes are involved in glycosylation pathway. The genes involved in the formation of aglycone are *sga*8, *sga*9, and *sga*10. The activator, Sga2, may control both the glycosylation system and the formation of aklavinone via polyketide pathway.

Sg4 derived from pSgc4 was cloned in the *Streptomyces* expression vector pIJE486 (Ylihonko *et al.,* 1996b) in *S. lividans* TK24 to give pSgs4. This vector is a high copy number plasmid that replicates in several *Streptomyces* spp. (Ward *et al.,* 1986) and it contains a constitutively expressed promoter, *erm*E (Bibb *et al.,* 1985) upstream from the multiple cloning site. The plasmid pSgs4 isolated from TK24 was introduced into the *S. galilaeus* strains that are blocked in deoxyhexose pathway of aclacinomycin biosynthesis and into the *S. peucetius* mutants producing ε–rhodomycinone based on a lesion in glycosylation genes. The ability of aclacinomycin production was restored by three *S. galilaeus* mutants, H063, H054 and H065. The mutant strain H063 accumulates aklavinone and it was completely complemented by the plasmid pSgs4. Instead, H054 and H065 producing aklavinone glycosides sharing neutral sugars, but not rhodosamine, were only partially complemented by pSgs4. Surprisingly, H063 carrying pSgs4 (H063/pSgs4) was able to produce aclacinomycins two–fold to that of the wild type *S. galilaeus*. *S. peucetius* M18 and M90 which produce ε–rhodomycinone were selected to hosts for pSgs4. L–rhamnosyl–ε–rhodomycinone (El Khamed *et al.*, 1977) was obtained when pSgs4 was expressed in the mutants M18 and M90 and, in addition, M18/pSgs4 produced L–daunosaminyl–ε–rhodomycinone (Essery and Doyle, 1980). The structures were not new ones but this demonstrates the ability of the gene cluster according to the present invention to generate hybrid products in a heterologous host. To produce hybrid compounds we prefer to use E1 medium supplemented with a suitable antibiotic, in this case, thiostrepton, to maintain the selection pressure for the plasmid containing strains. The products were extracted by organic solvents and purified by chromatography to obtain the compounds in high purity for structural elucidation.

Examples to further illustrate the invention are given hereafter.

Brief description of the drawings

FIG. 1 shows the structures of aclacinomycin, daunomycin and ε–rhodomycinone.

FIG. 2 is a diagram of the gene cluster for aclacinomycin biosynthesis.

FIG. 3 describes the proposed biosynthesis pathway for sugars found in aclacinomycins.

FIG. 4 shows the structures of the hybrid compounds produced by M18/pSgs4 (**1** and **2**) and M90/pSgs4 (**2**).

EXPERIMENTAL

Materials used

Restriction enzymes used were purchased from Promega (Madison,Wisconsin, USA), Fermentas (Lithuania) or Boehringer Mannheim (Germany), alkaline phosphatase from Boehringer Mannheim, and used according to manufacturers' instructions. Proteinase K was purchased from Promega and lysozyme from Sigma. Hybond™–N nylon membranes used in hybridization were purchased from Amersham (Buckinghamshire, England), DIG DNA Labelling Kit and DIG Luminescent Detection Kit from Boehringer Mannheim. Qiaquick Gel Extraction Kit from Qiagen (Hilden, Germany) was used for isolating DNA from agarose.

Bacterial strains and their use

Escherichia coli XL1BlueMRF' (Stratagene, La Jolla, California) was used for cloning.

Streptomyces lividans TK24 was the first cloning host for gene expression. The strain was provided by prof. Sir David Hopwood, John Innes Centre, UK.

The wild type, *Streptomyces galilaeus* ATCC 31615, produces aclacinomycins. It was used here to donate the genes of the invention.

Streptomyces galilaeus H039 (Ylihonko *et al.,* 1994) produces Akv–$(Rho)_{0-3}$. It was used as an expression host for pSgs4 being more easily transformed than the other mutants or the wild type.

Streptomyces galilaeus H054 (Ylihonko *et al.,* 1994) produces Akv–Rho–dF–$(CinA)_{0-1}$, Akv–dF–dF–$(CinA)_{0-1}$ and Akv–dF–Rho–Rho. It was used as an expression host for pSgs4.

Streptomyces galilaeus H063 produces aklavinone. It is a mutant strain derived from the wild type *S. galilaeus*. H063 was used as an expression host for pSgs4.

Streptomyces galilaeus H065 produces aklavinone with neutral glycosides. It is a mutant strain derived from the wild type *S. galilaeus*. H065 was used as an expression host for pSgs4.

Streptomyces peucetius M18 and M90 producing ε–rhodomycinone are the mutants derived from *S. peucetius* var. *caesius* (ATCC 27952). They were used as expression hosts for pSgs4.

Plasmids

E. coli cloning vectors pBluescript SK (Stratagene) and pUC19 (Pharmacia, Sweden) were used for making the subclones for sequencing and pBluescript was used also as a vector of a gene library.

pWHM1109 (provided by prof CR Hutchinson, Wisconsin, USA) is a shuttle vector replicating in *E. coli* and in streptomycetes. It was used as a vector of a gene library.

pIJ486 is a high copy plasmid vector provided by prof. Sir David Hopwood, John Innes Centre, UK (Ward *et al.,* 1986).

pIJE486 (Ylihonko *et al.*, 1996b) is an expression vector containing *erm*E (Bibb *et al.*, 1985) to promote expression of the cloned genes.

Nutrient media and solutions

For cultivation of *S. galilaeus* for total DNA isolation TSB medium was used. Lysozyme solution (0.3 M sucrose, 25 mM Tris, pH 8 and 25mM EDTA, pH 8) was used to isolate total DNA. TE buffer (10 mM Tris, pH 8.0 and 1mM EDTA) was used to dissolve DNA.

TRYPTONE-SOYA BROTH (TSB)

Per litre: Oxoid Tryptone Soya Broth powder 30 g.

ISP4

Bacto ISP-medium 4, Difco; 37 g/l.

E1 Per litre in tap water:

glucose	20 g
soluble starch	20 g
Farmamedia	5 g
Yeast extract	2.5 g
$K_2HPO_4 \circ 3H_2O$	1.3 g
$MgSO_4 \circ 7H_20$	1 g
NaCl	3 g
$CaCO_3$	3 g

pH adjusted to 7.4 before autoclaving

General methods:

NMR data was collected with a JEOL JNM-GX 400 spectrometer. ^1H and ^{13}C NMR samples were internally referenced to TMS.

The anthracycline metabolites were determined by (i) HPLC (LaChrom, Merck Hitachi, pump L-7100, detector L-7400 and integrator D-7500) using a LiChroCART RP-18 column. Acetonitrile:potassium hydrogen phosphate buffer (60 mM, pH 3.0 adjusted

with citric acid) was used as a mobile phase. Gradient system starting from 65 % to 30 % of potassium dihydrogen phosphate buffer was used to separate the compounds. The flow rate was 1 ml/min and the detection was carried out at 480 nm, and (ii) by TLC using precoated Kieselgel 60 F_{254} glass plates (Merck, Darmstadt, Germany) with an elution solution of toluene:ethyl acetate:methanol:formic acid (50:50:15:3).

ISP4 plates supplemented with thiostrepton (50 μg/ml) were used to maintain the plasmid carrying cultures.

Example 1. Cloning the gene cluster for aclacinomycin biosynthesis

1.1 Selection of clones by hybridization

For isolation of total DNA, *Streptomyces galilaeus* was grown for four days in 50 ml of TSB medium supplemented with 0.5% glycine. The cells were harvested by centrifuging for 15 min (3900 x g) in 12 ml Falcon tubes, and stored at −20°C. Cells from a 50 ml culture were used to isolate DNA. 5 ml of lysozyme solution containing 5 mg/ml of lysozyme was added on the cells of each Falcon tube, and incubated for 20 min at 37°C. 500 μl of 10% SDS containing 0.7 mg of proteinase K was added on the cells, and incubated for 80 min at 62°C, another 500 μl of 10% SDS containing 0.7 mg of proteinase K was added, and incubation was continued for 60 min. The sample was chilled on ice and 600 μl of 3M NaAc, pH 5.8 was added, and the mixture was extracted with equilibrated phenol (Sigma). The phases were separated by centrifuging (1400 x g) for 10 min. The DNA was precipitated from the water phase with an equal volume of isopropanol and collected by spooling with a glass rod and washed by dipping into 70% ethanol, air dried and dissolved in 500 μl of TE–buffer.

Southern hybridization to determine suitable restriction enzymes for preparing the restricted plasmid libraries was carried out using *Bgl*II, *Xho*I, *Not*I and their combinations. A fragment of about 9 kb hybridizing with the Sg–dht probe was preferred. For hybridization 600 ng of digested *S. galilaeus* DNA was loaded onto the agarose gel and after electrophoresis, the DNA was transferred from the gel to a nylon membrane by vacuum blotting. Hybridization was carried out according to Boehringer Mannheim's

manual 'The DIG System User's Guide for Filter Hybridization'. The probe for hybridization, Sg–dht, which was used for colony hybridization as well, was obtained by amplifying a gene fragment from the *S. galilaeus* DNA which is internal to the 4,6–dehydratase gene and corresponds to the fragment of 6345 to 6861 shown in SEQ ID NO:14. PCR was used for amplification, and the sequences for the degenerated oligonucleotide primers were 5'–CSGGSGSSGCSGGSTTCATSGG–3' (forward, SEQ. ID. NO:15) and 5'–GGGWRCTGGYRSGGSCCGTAGTTG–3' (reverse, SEQ. ID. NO:16). Suitable fragments were a 9 kb *Bgl*II fragment and a 7 kb *Xho*I–*Not*I fragment.

Ten micrograms of the chromosomal DNA was digested with *Bgl*II. The DNA fragments were separated by agarose gel electrophoresis and the band of 8 to 9 kb were cut from the 0.6% low gelling temperature SeaPlaque® agarose. The DNA band was isolated from the gel using Qiagen Gel Extraction Kit. The isolated fragment was ligated to pWHM1109 plasmid vector digested with *Bam*HI and defosforylated, in the ratio of 3 moles of the insert DNA to 1 mole of the vector DNA. The ligated DNA was introduced into *E. coli* XL1BlueMRF' by electroporation. Using the whole ligation mixture 786 colonies were obtained. The colonies were grown on agar plates for at least 12 h and transferred to nylon membranes. Hybridization of colony membranes was carried out as Southern using Sg–dht as a probe. Six clones gave signal in hybridization and the corresponding colonies were plated on agar and inoculated in 3 ml of LB medium for isolation of the plasmid DNA. Southern hybridization was used to study whether the plasmids derived from the clones carried the desired insert. Four of these plasmids contained the 4,6–dehydratase gene fragment and gave the identical restriction map thus carrying the same fragment representing both orientations. The fragment was designated as Sg4 and the plasmid containing the fragment as pSgc4.

In the same manner the plasmid library representing a 7 kb *Xho*I–*Not*I DNA fragment derived from *S. galilaeus* was constructed. pBluescript was digested with *Xho*I–*Not*I and the library containing the gene fragments of around 7 kb was constructed. In total 1523 colonies were hybridized and seven turned to be the desired clone. As described above, the clones were studied for the *Xho*I–*Not*I fragment. The insert fragment was designated as Sg5 and the plasmid as pSgc5. The strain *E. coli* XL1Blue MRF'/pSgc5 obtained was deposited according to the rules of the Budapest Treaty at Deutsche Sammlung von

Mikroorganismen und Zellkulturen GmbH (DSMZ) on August 12, 1999 with the accession number DSM 12999. The fragments Sg4 and Sg5 overlap within 836 bp corresponding bases from 6181 to 7016 in SEQ ID NO:14.

1.2. Subcloning the fragments for sequencing

To determine the nucleotide sequence of the whole cluster of the Sg4 and Sg5 suitable subclones were constructed. The convenient restriction sites were used for subcloning the 14806 bp region in the plasmids pUC19 and pBluescript. Nineteen subclones were needed to sequence Sg4, and 11 subclones for Sg5.

E. coli XL1BlueMRF' cells containing the subcloned plasmids were cultivated over-night at 37°C in 5 ml of LB–medium supplemented with 50 µg/ml of ampicillin. To isolate plasmids for sequencing reactions Wizard Plus Minipreps DNA Purification System kit of Promega or Biometra Silica Spin Disc Plasmid DNA Miniprep kit of Biomedizinische Analytik Gmbh were used according to the manufacturers' instructions.

DNA sequencing was performed using the automatic ABI DNA sequencer (Perkin–Elmer) according to the manufacturer's instructions.

1.3 Sequence analysis and the deduced functions of the genes

Sequence analyses were made using the GCG sequence analysis software package (Version 8; Genetics Computer Group, Madison, Wis., USA). The translation table was modified to accept also GTG as a start codon. Codon usage was analyzed using published data (Wright and Bibb 1992).

According to the CODONPREFERENCE program the sequenced DNA fragment revealed 11 complete open reading frames (ORFs), and two 5' ends of the other ORFs (*sga*1 and *sga*13). The functions of the genes were concluded by comparing the amino acid sequences translated from their base sequences to the known sequences in the data banks. The results are shown in Table 1 referring to the sequence data given in the application.

The suggested functions for the genes match well with a proposed biosynthetic pathway of sugars of aclacinomycins (Fig. 3). The last residue in a trisaccharide moiety of aclacinomycins is rhodinose that is enzymatically converted to cinerulose. Aclacinomycin N, a precursor of aclarubicin, contains rhodinose as the third sugar residue.

Table 1.

Gene	Position	Amino acids	Deduced function	Remarks
*sga*1	-1986 compl	>662	unknown	not complete Seq.ID.NO:1
*sga*2	2523-3341	272	activator	Seq.ID.NO:2
*sga*3	3355-4659 compl	434	dehydratase	Seq.ID.NO:3
*sga*4	4821-5810	329	oxidoreductase	Seq.ID.NO:4
*sga*5	5920-6891 compl	323	dTDP-glucose 4,6-de-hydratase	Seq.ID.NO:5
*sga*6	6879-8210 compl	443	glycosyl transferase (GTF)	Seq.ID.NO:6
*sga*7	8287-9618 compl	443	putative isomerase	Seq.ID.NO:7
*sga*8	9642-10445 compl	267	aklaviketone reductase (KRII)	Seq.ID.NO:8
*sga*9	10471-10905 compl	144	putative polyketide assembler	Seq.ID.NO:9
*sga*10	11115-11894	259	putative cyclase	Seq.ID.NO:10
*sga*11	11956-12672	238	aminomethylase	Seq.ID.NO:11
*sga*12	12685-13560 compl	291	glucose-1-phosphate thymidylyltransferase	Seq.ID.NO:12
*sga*13	13783-14805	341	aminotransferase	Seq.ID.NO:13 not complete

1.4 Expression cloning in Streptomyces strains

The 8 kb *Bam*HI-*Hind*III fragment from pSgc4 was ligated in pIJE486 to give pSgs4. Plasmid pSgs4 was introduced into *S. lividans* TK24 by protoplast transformation. The strain *S. lividans* TK24/pSgs4 obtained was deposited according to the rules of the

Budapest Treaty at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) on August 12, 1999 with the accession number DSM 12998. The plasmid pSgs4 was isolated from the strain, and further transferred into *S. galilaeus* mutant H039. The plasmid preparate isolated from H039 was subsequently introduced into H063, H054, and H065 mutants deficient of glycosylation system of aclacinomycins. The usage of H039 as a primary *S. galilaeus* host was due to the better efficiency for the intake of foreign DNA.

S. galilaeus mutants were studied for complementation by cultivating the clones containing pSgs4 in E1 medium supplemented with thiostrepton (10 μg/ml). The products from a 500 μl sample of the culture broth were extracted with toluene:methanol (1:1) at pH 7. The metabolites from both the transformed clones and the mutants were analyzed by TLC and HPLC to find the differencies caused by pSgs4. H063 producing endogenously aklavinone was restored to aclacinomycin producer with pSgs4. No aklavinone was found in the culture broth of H063/pSgs4. However, complementation was not completed when pSgs4 was expressed in H054 and H065. Both of the mutants produce aklavinone with neutral glycosides. Incomplete complementation was presumably due to the loss of the plasmids of some bacterial cells during cultivation, or a low expression of the genes needed as an activator is not present in pSgs4.

In the same manner, pSgs4 isolated from TK24 was introduced into the *S. peucetius* mutants M18 and M90. The characteristic product for these mutants is ε–rhodomycinone. The strains M18/pSgs4 and M90/pSgs4 containing the plasmid were cultivated in E1 medium supplemented with thiostrepton (10 μg/ml), and the metabolites therein were analyzed by TLC and HPLC. Both of the clones revealed an altered production profile as compared with the products obtained from the mutants. M90/pSgs4 accumulated a glycosylated product, yielding ε–rhodomycinone as the aglycone. The compound was identified as L–rhamnosyl–ε–rhodomycinone which has been previously synthesized (CAS=63252–11–9) by El Khamed *et al.* (1977).

M18/pSgs4 produced two compounds differing from the parental strain. According to the HPLC and TLC data one compound was the same as was produced by M90/pSgs4,

L-rhamnosyl-ε-rhodomycinone, and the other one was L-daunosaminyl-ε-rhodomycinone, which was previously characterized by Essery and Doyle (1980).

Table 2: TLC and HPLC data of the hybrid products

Product	Rf-value	Retention time
ε-rhodomycinone	0.67	6.70
L-rhamnosyl-ε-rhodomycinone	0.38	5.00
L-daunosaminyl-ε-rhodomycinone	0.04	4.06

1.5 Applicability of pSgs4 for strain improvement

Since H063 was completely complemented by pSgs4, the production level of aminoglycosides was studied. For this purpose, H063/pSgs4, H063 and the wild type *S. galilaeus* were cultivated in E1 medium in the Erlenmeyer bottles for four days. Two samples of 2 ml from each culture were extracted first with toluene:methanol (1:1) in acidic conditions to remove the neutral glycosides and the aglycones. The extraction procedure was repeated until neutral glycosides and the aglycones had disappeared from the water phase. The amount of anthracycline metabolites in toluene phase was determined and is shown in Table 3. Aclacinomycins containing rhodosamine were extracted from the water phase by chloroform. Both toluene and chloroform extracts were analyzed by TLC and toluene phases contained mostly aklavinone and the degradative products. Chloroform phases contained mainly aminoglycosides, although minor amounts of the aglycones were also detected. Extracts were evaporated to dryness and subsequently dissolved into 1 ml of methanol. The amounts of anthracycline metabolites were detected by spectrophotometer at 430 nm. The amounts related to absorbance were calculated using an extinction coefficient of 13000. The results given as mg/l of cultivation broth are shown in Table 3. The production of aclacinomycins by H063/pSgs4 was at least twofold better than obtained by the wild type.

Table 3.

	Chloroform phase aminoglycoside fraction		Toluene phase aglycone fraction	
Sample	Absorbance	Concentration (mg/l)	Absorbance	Concentration (mg/l)
H063	0.401	12.6	2.956	92.3
H063/pSgs4	2.751	85.9	2.974	92.9
S. galilaeus	1.338	41.8	0.690	21.5

The ability to increase the yield of aclacinomycins by pSgs4 in the mutant H063 suggests that the genes according to the present invention are useful in strain improvement.

Example 2. Compounds generated by pSgs4

The seed culture, 180 ml of E1 culture of the plasmid containing strains, M18/pSgs4 or M90/pSgs4, was obtained by cultivating each of the strains in three 250 ml Erlenmeyer flasks containing 50 ml of E1−medium supplemented with thiostrepton (5 μg/ml) for four days at 30°C, 330 rpm. The combined culture broths (180 ml) were used to inoculate 13 l of E1−medium in a fermentor (Biostat E). Fermentation was carried out for five days at 28°C (330 rpm, aeration: 450 l/min).

The cells were harvested by centrifuging. 2.6 l of methanol was used to brake the bacterial cells. The anthracycline metabolites were extracted from methanol solution at pH 8 using 2 l of ethyl acetate and the extract was evaporated to dryness. The viscous residue was loaded onto a silica column of 4 × 10 cm and toluene:ethyl acetate:formic acid (50:50:3) with increasing amount of methanol was used as an eluent. Pure fractions were pooled and extracted with 1M phosphate buffer (pH 8.0) and water. Organic phase

was dried with anhydrous Na_2SO_4 and then treated with hexane to effect precipitation. Pure compounds appeared as red powders dried under vacuum.

Complete structural determination of the compounds were accomplished by NMR. Proton and carbon assignments were based on a conventional NOE difference, pHSQC and HMBC measurements. Connectivities in particular relied heavily on HMBC experiment.

As deduced from the data given in Table 4, the structures revealed were L–rhamnosyl–ε–rhodomycinone (**1**) and L–daunosaminyl–ε–rhodomycinone (**2**) shown in Figure 4.

Although these structures were not novel, the generation of the hybrid products by the genes involved in glycosylation portion of aclacinomycin biosynthesis well demonstrates that the genes of pSgs4 are functional and ready to use in drug discovery for finding novel molecules.

Deposited microorganisms

The following microorganisms were deposited according to the Budapest Treaty at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D–38124 Braunschweig, Germany.

Microorganism	**Accession number**	**Date of deposit**
S. lividans TK24/pSgs4	DSM 12998	12 August 1999
E. coli XL1BlueMRF'/pSgc5	DSM 12999	12 August 1999

Table 4. ^{1}H and ^{13}C chemical shifts of **1** ($DMSO_{d6}$) and **2** (trace of TFA in $DMSO_{d6}$) in 400 and 100 MHz respectively.

	1		2	
Site	^{1}H	^{13}C	^{1}H	^{13}C
1	7.74, 1H, dd, 7.5, 0.9	118.9(d)	7.74, 1H, dd, 7.5, 1.0	119.7(d)
2	7.64, 1H, dd, 8.4, 7.5	136.5(d)	7.68, 1H, dd, 8.1, 7.5	137.4(d)
3	7.22, 1H, dd, 8.4, 0.9	124.1(d)	7.24, 1H, dd, 8.1, 1.0	125.0(d)
4	–	161.8(s)	–	162.6(s)
4–OH	12.00, 1H, s	–	exchange broadened	–
4a	–	115.2(s)	–	115.9(s)
5	–	189.9(s)	–	190.6(s)
5a	–	110.4(s)	–	111.4(s)
6	–	156.2(s)	–	157.1(s)
6–OH	13.41, 1H, s	–	exchange broadened	–
6a	–	135.1(s)	–	135.7(s)
7	5.14, 1H, d, 4.5	70.9(d)	5.15, 1H, d, 3.6	71.3(d)
8A	2.31, 1H, d, 15.1	28.9(t)	2.33, 1H, d, 14.6	34.0(t)
8B	2.14, 1H, dd, 15.1, 4.5	–	2.21, 1H, dd, 14.6, 3.8	–
9	–	70.0(s)	–	70.9(s)
10	4.16, 1H, s	51.2(d)	4.23, 1H, s	51.8(d)
10a	–	134.8(s)	–	136.1(s)
11	–	156.0(s)	–	156.8(s)
11–OH	12.77, 1H, s	–	exchange broadened	–
11a	–	110.8(s)	–	111.1(s)
12	–	185.4(s)	–	186.0(s)
12a	–	132.6(s)	–	133.3(s)
13A	1.73, 1H, dq, 13.9, 7.4	31.7(t)	1.83, 1H, dq, 14.1, 7.3	32.0(t)
13B	1.38, 1H, dq, 13.9, 7.4	–	1.47, 1H, dq, 14.1, 7.3	–
14	1.05, 3H, t, 7.4	6.09(q)	1.13,3H, t, 7.3	6.90(q)
15	–	170.4(s)	–	171.1(s)
16	3.63, 3H, s	51.7(q)	3.70, 3H, s	52.3(q)
1′	5.28, 1H, brs	103.7(d)	5.52, 1H, d, 3.1	100.7(d)
2′	3.83, 1H, d, 5.2	70.9(d)	2.18, 2H, m	27.1(t)
3′	3.44, 1H, dd, 9.0, 5.2	70.8(d)	3.40, 1H, dd, 11.8, 5.1	55.5(d)
4′	3.41, 1H, dd, 9.1, 9.0	72.0(d)	3.98, 1H, brs	67.0(d)
5′	3.77. 1H, dq, 9.1, 6.2	68.9(d)	4.21, 1H, q, 6.3	65.3(d)
6′	1.29, 3H, d, 6.2	16.9(q)	1.32, 3H, t, 6.3	16.7(q)

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Ala Pro Asp His Phe Asp Leu Asp Arg Pro Ala Gly Gln Gly Gln Leu
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Ser Leu Ser Gly Pro His Thr Ala Leu Phe Gly Ala Phe Ala Arg Leu
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Gln Ala Glu Thr Ala Val Arg Thr Leu Arg Glu Arg Arg Pro Val Leu
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Ala Pro Ala Gly Ala Val Leu Arg Arg Met Arg Ser Pro Val Leu Gly
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Ala Val Leu Arg Phe Pro Leu Thr Thr Ser Ala
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<213> *Streptomyces galilaeus*

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Ile Gly Arg Ala Thr Ala His Ala Phe Ala Asp Arg Gly Asp Arg Val
35 40 45

Leu Val Val Gly Arg Thr Ala Ala Thr Leu Ala Gly Thr Ala Glu Gly
50 55 60

His Pro Gly Ile Ser Val Leu Thr Ala Asp Leu Thr Asp Pro Asp Gly
65 70 75 80

Pro Arg Ala Ile Thr Asp Ala Ala Leu Asp Ala Leu Gly Arg Ile Asp
85 90 95

Val Leu Val Asn Asn Ala Ala Thr Gly Gly Phe Ala Gly Leu Ala Glu
100 105 110

Thr Glu Pro Glu Ala Ala Arg Glu Gln Phe Asp Ser Asn Leu Leu Ala
115 120 125

Pro Leu Leu Leu Thr Arg Gln Thr Leu Asp Ala Leu Ser Ala Asp Gly
130 135 140

Gly Gly Thr Val Leu Asn Ile Gly Ser Ala Gly Ala Leu Gly Arg Arg
145 150 155 160

Ala Trp Pro Gln Asn Gly Val Tyr Gly Ala Ala Lys Ala Gly Leu Asp
165 170 175

Phe Leu Thr Arg Thr Trp Ala Val Glu Leu Ala Pro Arg Gly Ile Arg
180 185 190

Val Leu Gly Leu Ala Pro Gly Val Ile Asp Thr Gly Ile Gly Glu Arg
195 200 205

Ser Gly Met Ser Arg Glu Ala Tyr Ala Gly Phe Leu Gly Gln Ile Ala
210 215 220

Ala Arg Val Pro Ala Gly Arg Val Gly Arg Pro Glu Asp Ile Ala Trp
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Trp Ala Val Gln Leu Ala Asp Pro Arg Ala Ala Tyr Ala Thr Gly Ala
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Val Leu Ala Val Asp Gly Gly Leu Ser Leu Thr
260 265

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<212> PRT
<213> *Streptomyces galilaeus*

<400> 9
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Gln His Phe Tyr Ala Arg Gln Met Arg Tyr Leu Asp Ser Gly Glu Ala
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Glu Thr Trp Ala Gly Thr Phe Thr Glu Asp Gly Ser Phe Ala Pro Pro
35 40 45

Ser Leu Pro Glu Pro Val Arg Gly Arg Pro Leu Leu Ala Glu Gly Ala
50 55 60

Arg Asn Ala Ala Ala Gly Leu Ala Ala Ala Arg Glu Thr His Arg His
65 70 75 80

Trp Val Gly Met Leu Thr Val Thr Pro Ala Asp Asp Gly Ser Leu Thr
85 90 95

Ala Glu Ser Leu Val Ser Ile Val Ala Val Ala Gln Gly Gly Pro Ala
100 105 110

Arg Leu His Leu Val Cys Thr Cys Arg Asp Val Leu Val Arg Glu Gly
115 120 125

Gly Arg Leu Leu Val Arg Glu Arg Val Val Thr Arg Asp Asp Arg Pro
130 135 140

<210> 10
<211> 259
<212> PRT
<213> *Streptomyces galilaeus*

<400> 10
Val Arg Ile Ile Asp Leu Ser Ser Pro Val Asp Ala Ala Gly Phe Glu
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Pro Asp Pro Val Val His Asp Val Leu Gly Pro Lys Glu Ala Ala Thr
20 25 30

His Met Ser Glu Glu Met Arg Glu His Phe Gly Ile Asp Phe Asp Pro
35 40 45

Ala Glu Leu Pro Glu Gly Glu Phe Leu Ser Leu Asp Arg Leu Gln Leu
50 55 60

Thr Thr His Thr Gly Thr His Val Asp Ala Pro Ser His Tyr Gly Thr
65 70 75 80

Arg Ala Ala Tyr Arg Asp Gly Pro Pro Arg His Ile Asp Glu Met Pro
85 90 95

Leu Asp Trp Phe Phe Arg Pro Ala Val Val Leu Asp Leu Ser Asp Gln
100 105 110

Gly Thr Gly Ala Val Gly Ala Asp Val Leu Arg Arg Glu Met Asp Arg
115 120 125

Ile Gly His Thr Pro Ser Pro Met Asp Ile Val Leu Leu Arg Thr Gly
130 135 140

Ala Asp Ala Trp Ala Gly Thr Pro Lys Tyr Phe Thr Asp Phe Thr Gly
145 150 155 160

Leu Asp Gly Ser Ala Val His Leu Leu Leu Asp Leu Gly Val Arg Val
165 170 175

Ile Gly Thr Asp Ala Phe Ser Leu Asp Ala Pro Phe Gly Asp Ile Ile
180 185 190

Thr Arg Tyr Arg Ala Thr Gly Asp Pro Ser Val Leu Trp Pro Ala His
195 200 205

Val Ile Gly Arg Asp Arg Glu Tyr Cys Gln Val Glu Arg Leu Ala Gly
210 215 220

Leu Asp Arg Leu Pro Ala Ala His Gly Phe Arg Val Ala Cys Phe Pro
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Val Arg Ile Ala Gly Ala Gly Ala Gly Trp Thr Arg Ala Val Ala Leu
245 250 255

Val Asp Glu

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<211> 238
<212> PRT
<213> *Streptomyces galilaeus*

<400> 11
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Arg Gly Lys Asp Trp Gly Gln Glu Ala Ala Asp Val Ser Arg Ile Ile
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Thr Glu Arg Arg Pro Gly Ala Gly Ser Leu Leu Asp Val Ala Cys Gly
35 40 45

Thr Gly Ala His Leu Ser Val Phe Ser Thr Leu Phe Glu Val Ala Glu
50 55 60

Gly Leu Glu Ile Ala Glu Pro Met Arg Arg Leu Ala Glu Gln Arg Leu
65 70 75 80

Pro Gly Thr Thr Val His Ala Gly Asp Met Arg Asp Phe Arg Leu Pro
85 90 95

Arg Thr Tyr Asp Ala Val Ser Cys Met Phe Cys Ala Ile Gly Tyr Leu
100 105 110

Glu Thr Leu Asp Asp Met Arg Ala Ala Val Arg Ser Met Ala Ala His
115 120 125

Leu Glu Pro Gly Gly Val Leu Val Val Glu Pro Trp Trp Phe Pro Glu
130 135 140

Asn Phe Ile Glu Gly Tyr Val Ala Gly Asp Leu Ala Arg Glu Glu His
145 150 155 160

Arg Thr Ile Ala Arg Ile Ser His Thr Thr Arg Lys Gly Arg Ala Thr
165 170 175

Arg Met Glu Val Arg Phe Thr Val Gly Asp Ala Ala Gly Ile Gln Gln
180 185 190

Phe Thr Glu Ile Asp Val Leu Thr Leu Phe Thr Arg Asp Glu Tyr Thr
195 200 205

Ala Ala Phe Thr Asp Ala Gly Cys Ser Val Glu Phe Leu Glu Asp Gly
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Pro Thr Gly Arg Gly Leu Phe Val Gly Val Arg Glu Gln Arg
225 230 235

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<211> 291
<212> PRT
<213> *Streptomyces galilaeus*

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Met Ile Tyr Tyr Pro Leu Ser Val Leu Met Leu Ala Asp Ile Arg Glu
35 40 45

Ile Leu Leu Ile Cys Thr Glu Arg Asp Leu Glu Gln Phe Arg Arg Leu
50 55 60

Leu Gly Asp Gly Ser Gln Leu Gly Leu Arg Ile Asp Tyr Ala Val Gln
65 70 75 80

Asn Arg Pro Ala Gly Leu Ala Asp Ala Phe Val Ile Gly Ala Asp His
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Val Gly Asp Asp Asp Val Ala Leu Val Leu Gly Asp Asn Ile Phe His
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Gly His His Phe Tyr Asp Leu Leu Gln Ser Asn Val His Asp Val Gln
115 120 125

Gly Cys Val Leu Phe Gly Tyr Pro Val Glu Asp Pro Glu Arg Tyr Gly
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Val Gly Glu Thr Asp Ala Ser Gly Gln Leu Val Ser Leu Glu Glu Lys
145 150 155 160

Pro Leu Arg Pro Arg Ser Asp Leu Ala Ile Thr Gly Leu Tyr Leu Tyr
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Asp Asn Glu Val Val Asp Ile Ala Lys Asn Leu Arg Pro Ser Pro Arg
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Gly Glu Leu Glu Ile Thr Asp Val Asn Arg Asn Tyr Leu Ala Arg Gly
195 200 205

Arg Ala Arg Leu Val Asp Leu Gly Arg Gly Phe Ala Trp Leu Asp Ala
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Gly Thr Pro Glu Ser Leu Leu Gln Ala Thr Gln Tyr Val Arg Thr Leu
225 230 235 240

Glu Glu Arg Gln Gly Val Arg Ile Ala Cys Val Glu Glu Val Ala Leu
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Arg Met Gly Phe Ile Asp Ala Asp Met Cys His Arg Leu Gly Glu Gln
260 265 270

Met Ser Gln Ser Gly Tyr Gly Arg Tyr Val Met Ala Val Ala Arg Glu
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Phe Ser Gly
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<210> 13
<211> 341
<212> PRT
<213> *Streptomyces galilaeus*

<400> 13
Met Thr Thr Leu Val Trp Asp Tyr Leu Gln Glu Tyr Glu Asn Glu Arg
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Ala Asp Ile Leu Asp Ala Val Glu Thr Val Phe Ser Ser Gly Arg Leu
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Val Leu Gly Asp Ser Val Arg Gly Phe Glu Glu Glu Phe Ala Ala Tyr
35 40 45

His Gly Ala Ala His Cys Val Gly Val Asp Asn Gly Thr Asn Ala Ile
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Lys Leu Ala Leu Gln Ala Leu Gly Val Gly Pro Gly Asp Glu Val Val
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Thr Val Ser Asn Thr Ala Ala Pro Thr Val Val Ala Ile Asp Ser Val
85 90 95

Gly Ala Thr Pro Val Phe Val Asp Val His Pro Asp Ser Tyr Leu Met
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Asp Thr Glu Gln Val Glu Ala Ala Leu Thr Pro Arg Thr Arg Cys Leu
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Leu Pro Val His Leu Tyr Gly Gln Cys Val Asp Leu Ala Pro Leu Glu
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Arg Leu Ala Ala Glu His Asp Leu Phe Leu Val Glu Asp Cys Ala Gln
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Ala His Gly Ala Arg Arg Ala Gly Arg Leu Ala Gly Thr Thr Gly Asp
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Ala Ala Ala Phe Ser Phe Tyr Pro Thr Lys Val Leu Gly Ala Tyr Gly
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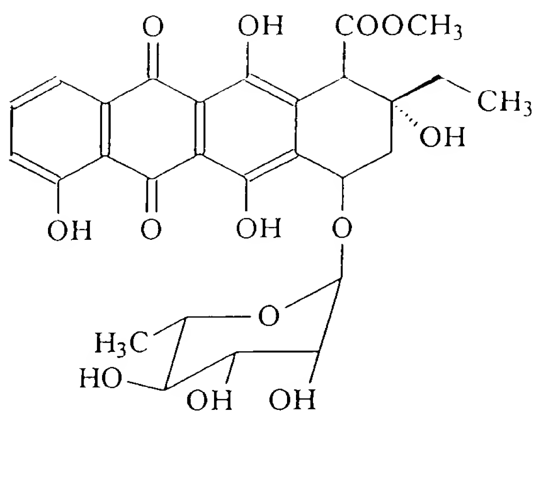
Figure 4



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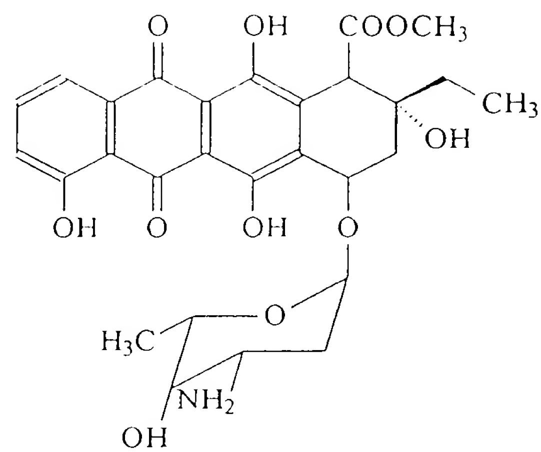

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